REMARKS

Reconsideration and withdrawal of the rejections of the application are requested in view of the herein amendments, arguments and submissions.

I. STATUS OF CLAIMS AND FORMAL MATTERS

Claim 22 is pending in this application. Disclosure regarding effective dosage amounts to be administered can be found in the section of the application beginning on page 26, line 10. No new matter is added.

It is submitted that the claims, herewith and as originally presented, are patentably distinct over the prior art cited by the Examiner, and that these claims are in full compliance with the requirements of 35 U.S.C. § 112. Amendments to the claims are not made for the purpose of patentability within the meaning of 35 U.S.C. §§ 101, 102, 103 or 112. Rather, these changes are made simply for clarification and to round out the scope of protection to which Applicants are entitled. Furthermore, it is explicitly stated that the amendments should not give rise to any estoppel, as they are not narrowing amendments.

II. THE REJECTION UNDER 35 U.S.C. § 112, 2ND PARAGRAPH, IS OVERCOME

Claim 22 was rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. Claim 22 is amended to specify that the soluble VEGF receptor and angiopoietin-1 are administered in an amount effective to reduce the risk of restenosis. As explained in the specification, effective dosage can be determined by the skilled practitioner depending on factors such as age, sex, weight, condition and nature of the patient. (See page 26, lines 15-18.) Reconsideration and withdrawal of the indefiniteness rejection is requested.

III. THE REJECTION UNDER 35 U.S.C. § 112, 1ST PARAGRAPH, IS OVERCOME

Claim 22 was rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. The rejection is traversed.

The Office Action alleges that there is no direct evidence to support the claim that administration of a soluble VEGF receptor and ang-1 reduces the risk of restenosis. It further states that since the result is unpredictable, undue experimentation would be required to practice the claimed method. The enclosed article by Nambu *et al.* (Gene Therapy, 2004 11:865-873) provides proof of principle supporting the present claim.

Nambu *et al.* examined ocular neovascularization, which is a model relevant to restenosis, as they are both characterized by excessive angiogenesis. They used a transgenic approach to evaluate the effects of overexpression of Angl (a vessel maturation inducer) in mice with ocular neovascularization. It is well known, at least in the ischemic model, that VEGF expression is markedly increased, and that VEGF is a key factor in the development of increased neovascularization.

The investigators demonstrated that increased expression of Angl in eyes with severe retinal ischemia or in eyes with rupture of Bruch's membrane significantly suppressed the development of retinal or choroidal neovascularization. Angl also significantly reduced VEGF-induced retinal vascular permeability.

These data show that the overexpression of Angl and the reduction of VEGF expression reduce neovascularization in these models. These results exactly parallel the disclosure of the present application. The Office Action claims that a model using gene therapy cannot be used to predict the effect of administering the protein encoded by the transgene. Applicants dispute this assertion. While it holds true that perhaps successful protein therapy may not predict successful gene therapy, the argument in the other direction is not tenable. One cannot extrapolate from protein therapy to gene therapy because of the potential problems with gene targeting and expression. However, if gene therapy has proven to be effective, it is because the protein is expressed and functional. The mere presence of the nucleic acid alone is generally not therapeutic (except in specific usages that are not relevant here). Therefore, if gene therapy is effective, it can reasonably be predicted that protein therapy will be effective as well, because it is the presence of the protein in both instances that is therapeutic.

The study by Nambu *et al.* demonstrates that VEGF inhibition, combined with induction of vessel maturation, prevents neovascularization. Neovascularization, or angiogenesis, occurs during restenosis. (See, for example, the paragraph bridging pages 1 and 2 and the last full paragraph on page 3 of the specification.) Therefore, preventing neovascularization by inhibiting VEGF and inducing vessel maturation, as disclosed in the present application and demonstrated by Nambu *et al.*, will reduce the risk of restenosis.

Reconsideration and withdrawal of the enablement rejection are requested.

CONCLUSION

In view of the remarks and amendments herewith, the application is believed to be in condition for allowance. Favorable reconsideration of the application and prompt issuance of a Notice of Allowance are earnestly solicited. Alternatively, at least withdrawal of finality of the Office Action issued on November 3, 2004 is requested, as finality was inappropriate in view of the new rejection under 35 U.S.C. § 112, second paragraph, which was not necessitated by any amendments made by Applicants. The undersigned looks forward to hearing favorably from the Examiner at an early date.

Respectfully submitted,

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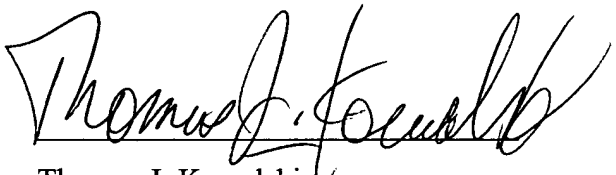
Attorneys for Applicants



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Gene Therapy (2004) 11, 865–873

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RESEARCH ARTICLE

Angiopoietin 1 inhibits ocular neovascularization and breakdown of the blood–retinal barrier

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Several retinal and choroidal diseases are potentially treatable by intraocular delivery of genes whose products may counter or neutralize abnormal gene expression that occurs as part of the diseases. However, prior to considering a transgene, it is necessary to thoroughly investigate the effects of its expression in normal and diseased eyes. An efficient way to do this is to combine tissue-specific promoters with inducible promoter systems in transgenic mice. In this study, we used this approach to evaluate the effects of ectopic expression of angiopoietin-1 (Ang1) in normal eyes and those with ocular neovascularization. Adult mice with induced expression of Ang1 ubiquitously, or specifically in the retina, appeared normal and had no identifiable changes in retinal or choroidal blood vessels or in retinal function as assessed by electroretinography. Increased expression of Ang1 in eyes with severe retinal ischemia or in eyes with rupture of Bruch's membrane significantly suppressed the development of retinal or choroidal neovascularization, respectively. This inhibition of ocular neovascularization is particularly interesting and noteworthy, because overexpression of Ang1 in skin stimulates neovascularization. Ang1 also significantly reduced VEGF-induced retinal vascular permeability. These data suggest that intraocular delivery of ang1 has potential for treatment of ocular neovascularization and macular edema.

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Keywords: *Ang1; antiangiogenesis; blood–retinal barrier; choroidal neovascularization; diabetic retinopathy; gene delivery; macular edema; retinal neovascularization*

Introduction

Angiogenesis is essential during development of vertebrate animals and disruption of genes that regulate angiogenesis often leads to embryonic lethality. In fact, VEGF-A is so critical that disruption of only one copy of the *vegfa* gene is lethal.[1,2] VEGF-A is needed for differentiation of endothelial cells and formation of tubules. Several gene products play a role in maturation and remodeling of endothelial tubules into stable blood vessels, including the endothelial specific receptor, Tie2, and its binding partners, the angiopoietins. Mice with targeted disruption of *Tie2* die between E9.5 and E12.5 showing lack of development of the endothelial lining of the heart, lack of remodeling of the primary capillary plexus to form more complex higher order branching vessels, failure of vascular invasion of neuroectoderm, and lack of recruitment of periendothelial cells.[3,4] Angiopoietin-1 (Ang1), which binds with high affinity to Tie2 and initiates phosphorylation and down stream signaling,[5] is a critical Tie2 agonist; mice deficient in Ang1 die around E12.5 and show vascular defects similar to, but less severe than, those seen in Tie2-deficient mice.[6] Angiopoietin-2 (Ang2) also binds with high affinity to Tie2, but does not stimulate its phosphorylation in cultured endothelial cells.[7] During embryonic vascular development, Ang2 acts as an Ang1 antagonist; transgenic mice overexpressing Ang2 have a phenotype similar to Ang1-deficient mice.[7]

The functions of Ang1 and Ang2, and the manner in which Ang/Tie2 signaling modulates VEGF signaling after embryonic development is less clear. Ang1 is ubiquitously expressed in association with adult blood vessels, while Ang2 is expressed predominantly in tissues such as the ovary where there is vascular turnover.[7] This has led to the hypothesis that Ang1 is a critical maintenance factor that stabilizes blood vessels, and that Ang2 acts as a competitive antagonist of Ang1 and destabilizes blood vessels when angiogenesis is needed. This hypothesis, although attractive, has been difficult to test. One technical problem has been that Ang1 forms multimers, which complicates its isolation and use for *in vitro* studies.[8] A genetically engineered chimeric protein, Ang1*, which does not

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form large multimers but still activates Tie2 receptors, has allowed investigation of the effects of activating Tie2 in various *in vitro* systems.[9] Ang1* promotes survival of cultured endothelial cells and stabilizes tubular networks.[10–12] Since Ang1* does not behave exactly like Ang1, it cannot be concluded that Ang1 has the same functions, but such studies at least provide some support for a potential Ang1 vascular maintenance function. However, there is also evidence suggesting that Ang1 may stimulate chemotaxis, tubule formation, and vascular sprouting and hence act as a proangiogenic agent.[9,13–15] In fact, transgenic mice with increased expression of Ang1 in skin under control of the keratin-14 promoter show a moderate increase in number and a large increase in diameter of dermal vessels, demonstrating that Ang1 is proangiogenic in skin.[16] Transgenic mice with increased expression of VEGF in skin showed a large increase in leaky dermal vessels, and double transgenic mice with coexpression of Ang1 and VEGF had an additive effect on angiogenesis, but the vessels did not leak spontaneously and were resistant to inflammation-induced leakage.[17] Increased expression of Ang1 in adult mice by intravascular injection of adenoviral vectors expressing Ang1 did not cause increased vascularity, but rather conferred leakage resistance to existing vessels.[18] Therefore, the effects of Ang1 may vary depending upon the setting.

If Ang1 stabilizes retinal blood vessels, it may provide benefit in several diseases of the retina and choroid complicated by excessive vascular permeability, but a proangiogenic effect could be deleterious. In this study, we sought to assess the effects of Ang1 in the retina and investigate its potential as a therapeutic transgene by generating double transgenic mice with ubiquitous or retina-specific, doxycyline-inducible expression of Ang1.

Results

Double transgenic mice with inducible expression of Ang1 in the retina

The doxycycline inducible promoter system in transgenic mice is a very useful tool, which in past studies has allowed detailed exploration of the effects of VEGF[19,20] and brain-derived neurotrophic factor in the retina.[21] It requires generation of two transgenic mice, one in which reverse tetracycline transactivator is coupled to a promoter that drives its expression ubiquitously or in a certain tissue. In this study, we used the opsin promoter for photoreceptor-specific expression and the CMV promoter for ubiquitous expression. A construct in which the tetracycline response element is coupled to the gene of interest, in this case *ang1*, is used to generate the second transgenic. The two types of transgenics are crossed to yield TetOpsinAng1 or TetCMVAng1 double transgenics. When not treated with doxycyline, adult TetOpsinAng1 and TetCMVAng1 mice showed low level *ang1* mRNA in the retina similar to that seen in wild-type mice (Figure 1). After treatment with 2 mg/ml of doxycycline in drinking water for 2 weeks, both types of double transgenic mice showed a large increase in *ang1* mRNA (Figure 1). Immunostaining for Ang1 in wild-type mice and in TetOpsinAng1 or TetCMVAng1 mice in the absence of doxycycline, showed faint, barely detectable staining in the retina (Figure 2a, c, e, respectively). In the absence of primary antibody, the faint staining was not observed (Figure 2b). After 2 weeks of 2 mg/ml of doxycycline in drinking water, TetOpsinAng1 (Figure 2d) and TetCMVAng1 (Figure 2f) mice showed increased staining for Ang1 that was most prominent in the outer nuclear layer, but there was also slightly increased staining in the inner retina.

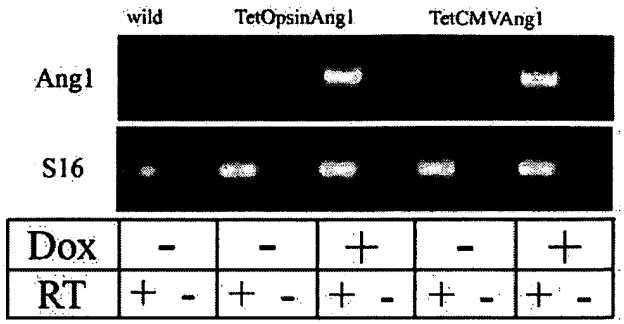

Figure 1 Expression of ang1 mRNA in double transgenic mice. Adult TetOpsinAng1 or TetCMVAng1 mice were treated with 2 mg/ml of doxycycline in their drinking water (Dox +) or given normal drinking water (Dox −). After 2 weeks, retinal RNA was isolated and reverse transcriptase (RT)-PCR was performed using primers specific for ang1 and mRNA for S16 ribosomal protein. In the absence of doxycycline, TetOpsinAng1 and TetCMVAng1 mice had low levels of ang1 mRNA in the retina, comparable to that seen in wild-type mice. When treated with doxycycline, TetOpsinAng1 and TetCMVAng1 mice each showed substantial induction of ang1 mRNA expression. In the absence of RT, no mRNA was seen.

Onset of increased expression of Ang 1 in adulthood has no effect on retinal or choroidal vessels

Staining with the vascular marker, GSA, shows blood vessels on the surface of the retina and in intermediate and deep capillary beds in adult TetOpsinAng1 (Figure 2g) and TetCMVAng1 (Figure 2h) mice that were not treated with doxycycline. After 2 or 8 weeks of 2 mg/ml of doxycycline in drinking water, TetOpsinAng1 (Figure 2i and k) or TetCMVAng1 mice (Figure 2j and l) had normal appearing retinas and retinal blood vessels. Compared to untreated double hemizygous mice, there was no significant difference in ERG a-wave or b-wave amplitudes in mice that had had increased expression of Ang1 in the retina for 8 weeks (Figure 3).

Increased expression of Ang1 supresses retinal neovascularization in mice with ischemic retinopathy

In neonatal mice, hyperoxia induces large areas of nonperfused retina due to occlusion of retinal blood vessels, resulting in ischemia-induced retinal neovascularization.[22] This model is relevant to proliferative diabetic retinopathy and other retinal vascular occlusive diseases such as branch and central retinal vein occlusions. Untreated P17 double hemizygous TetCMVAng1 mice with ischemic retinopathy showed extensive retinal neovascularization (Figure 4a) like that typically seen in wild-type mice with ischemic retinopathy. In contrast, TetCMVAng1 mice treated with doxycycline to induce expression of Ang1 during the ischemic period between

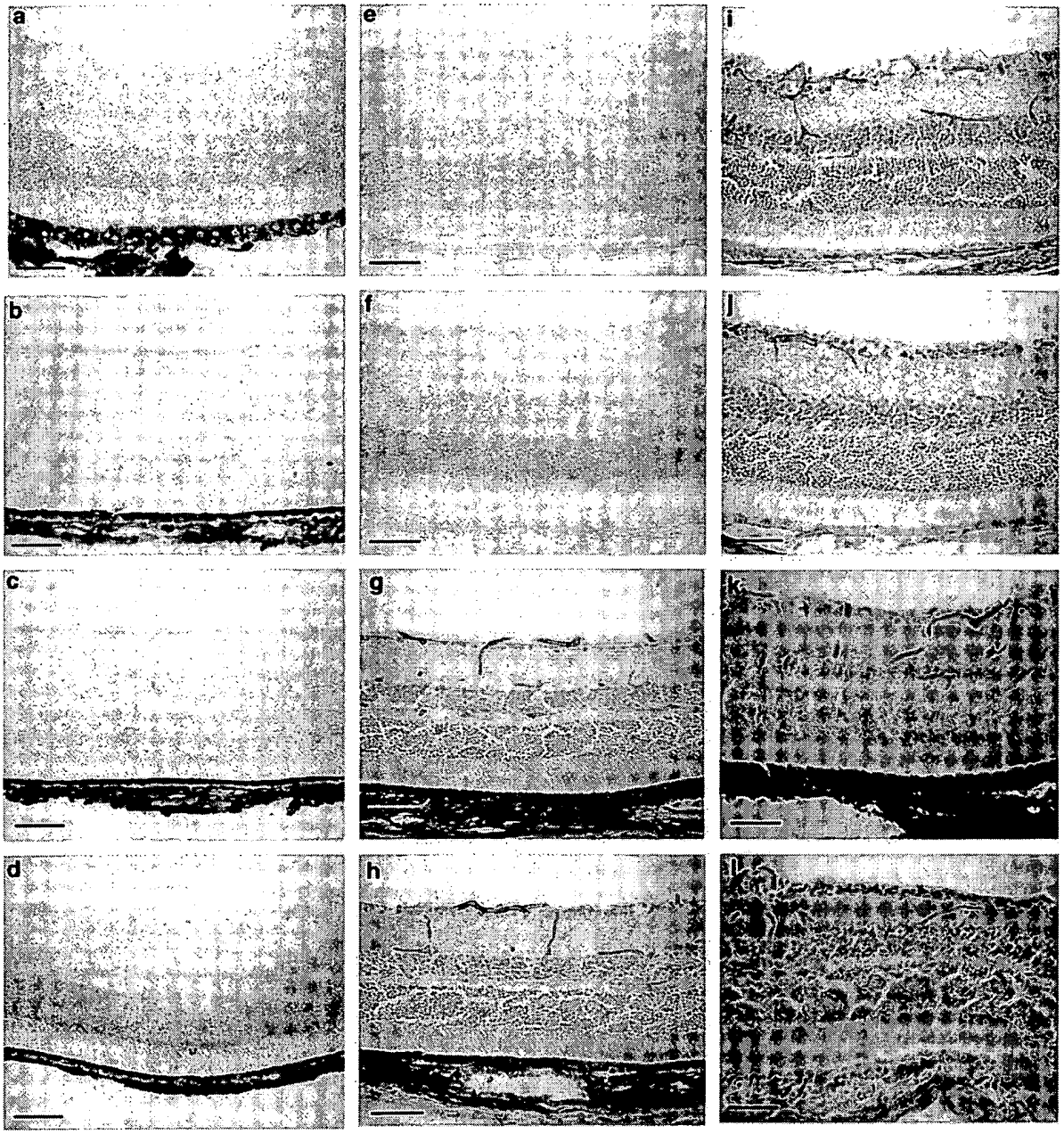

Figure 2 Adult mice with increased expression of Ang1 have normal appearing retinas with no identifiable change in retinal vessels. Immunohistochemical staining for Ang1 in wild-type mice showed faint, barely detectable red reaction product in the retina (a). In the absence of primary antibody, there was complete absence of staining (b). In the absence of doxycycline, TetOpsinAng1 (c) and TetCMVAng1 mice (e) showed faint staining in the retina similar to that seen in wild-type mice (a). After 2 weeks of 2 mg/ml of doxycycline in drinking water, TetOpsinAng1 (d) and TetCMVAng1 mice (f) showed increased red reaction product predominantly in photoreceptors, and also in the inner retina. Staining with the vascular marker, Griffonia simplicifolia *lectin, showed blood vessels on the surface of the retina and in intermediate and deep capillary beds in adult TetOpsinAng1 (g) and TetCMVAng1 (h) mice that were not treated with doxycycline. After 2 or 8 weeks of 2 mg/ml of doxycycline in drinking water, TetOpsinAng1 (i and k) or TetCMVAng1 mice (j and l) still showed normal appearing retinas and retinal blood vessels. Bar = 50 μm.*

P12 and P17 showed very little retinal neovascularization (Figure 4b). Image analysis showed that doxycycline-treated TetCMVAng1 mice with ischemic retinopathy had significantly less retinal neovascularization above the internal limiting membrane than doxycycline-treated control mice with ischemic retinopathy (Figure 4c). The TetCMVAng1 mice had greater levels of *ang1* mRNA, but no difference in *vegf* mRNA compared to the control mice (Figure 4d).

Ang1 suppresses the development of choroidal neovascularization at rupture sites in Bruch's membrane

Choroidal neovascularization has not been clearly linked to ischemia or hypoxia and occurs in different settings than retinal neovascularization. It occurs as a complication of diseases of the Bruch's membrane/retinal pigmented epithelium complex, such as age-related

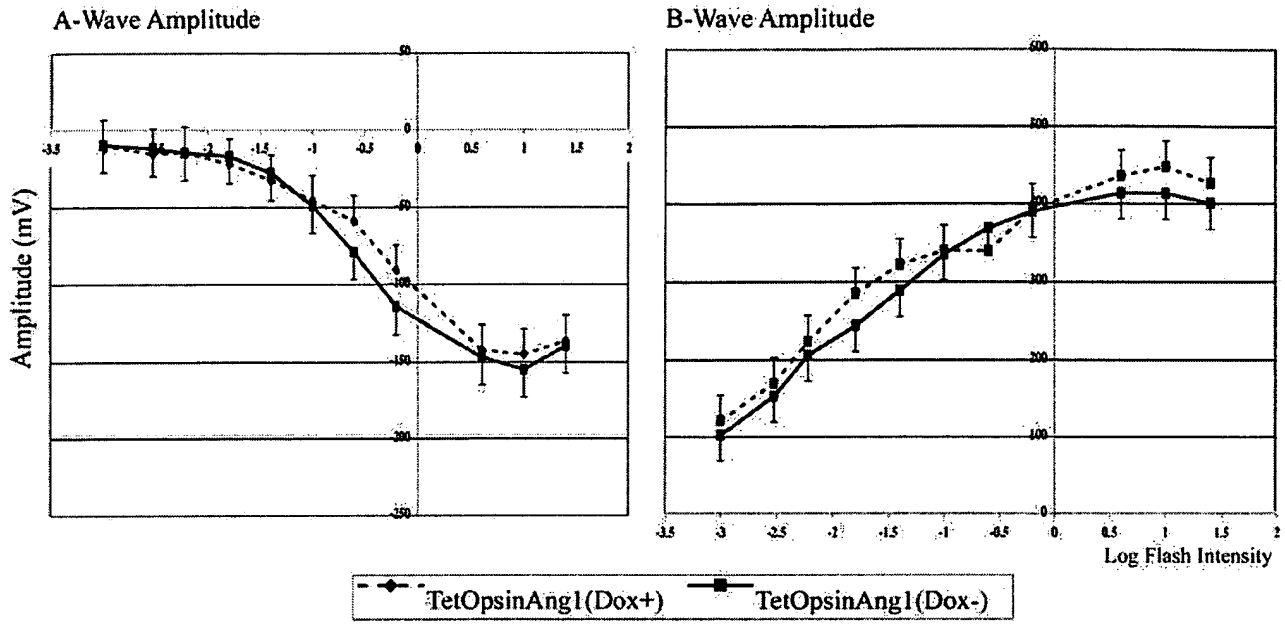

Figure 3 Increased expression of Ang2 in the retina for 8 weeks does not alter retinal function assessed by electroretinograms (ERGs). TetOpsinAngl mice were given normal drinking water (Dox−, n = 4) or drinking water containing 2 mg/ml of doxycycline (Dox +, n = 4) for 8 weeks and then mice were dark-adapted and ERGs were performed as described in Materials and methods. Repeated measures ANOVA showed no significant differences in ERG a-wave (P = 0.570) or b-wave amplitudes (P = 0.345) between mice with ectopic expression of Angl in the retina for 8 weeks and mice with normal endogenous expression of Angl.

macular degeneration. It also occurs in diseases in which there are ruptures or cracks in Bruch's membrane such as high myopia and traumatic rupture of Bruch's membrane. Laser-induced rupture of Bruch's membrane provides a useful model of choroidal neovascularization in mice[23] and this model was used to assess the effect of increased expression of Angl on choroidal neovascularization. Bruch's membrane was ruptured in three locations in each eye of adult TetCMVAngl mice. Controls were given normal drinking water and the experimental group was treated with 2 mg/ml of doxycycline in their drinking water. After 2 weeks, the mice were perfused with fluorescein-labeled dextran and ruture sites were examined on choroidal flat mounts by fluorescence microscopy. Doxycycline-treated TetCMVAngl mice had significantly less choroidal neovascularization than controls (Figure 5).

Angl suppresses VEGF-induced breakdown of the blood–retinal barrier

Diabetic macular edema is a major cause of visual morbidity. Recently, signaling through VEGF receptors has been implicated in the pathogenesis of diabetic macular edema.[24] One way to model VEGF-induced breakdown of the blood–retinal barrier is to inject VEGF into the vitreous cavity, which results in prominent vascular leakage that is easily assessed using [^{3}H]mannitol as a marker.[25] Adult TetOpsinAngl mice or littermate controls that carried only one of the transgenes were treated with 2 mg/ml of doxycycline for 2 weeks, and then given an intravitreous injection of 1 µl of 10^{-6} M VEGF. After 6 h, the retina to lung and retina to kidney leakage ratios were significantly less in TetOpsinAngl mice (Figure 6).

Discussion

Angl plays a critical role during embryonic development, but its function in adults is unclear. It may act as a vascular stabilizing agent,[9–12] but in skin, increased expression of Angl stimulates the growth of blood vessels.[16,17] In this study, we investigated the effects of Angl in the retina. Unlike the situation in skin, increased expression of Angl in the retina does not cause an increase in the number or size of retinal vessels. Increased expression of Angl for up to 8 weeks had no identifiable effect on the structure of retinal or choroidal vessels, and retinal function as assessed by ERG was normal. Despite the unchanged appearance of retinal vessels at the light microscopic level, Angl decreased VEGF-induced leakiness of the vessels. Angl also inhibited ischemia-induced neovascularization in the retina, and suppressed the development of choroidal neovascularization at Bruch's membrane rupture sites. Therefore, in the eye, Angl exhibits antiangiogenic and antipermeability effects.

Patients with diabetic retinopathy have increased permeability of retinal vessels resulting in moderate vision loss from macular edema, and develop retinal neovascularization that leads to severe vision loss from scarring and retinal detachment. Early in their course, patients with choroidal neovascularization experience loss of vision due to excessive permeability of the new vessels resulting in collection of fluid beneath the retina. Over time, choroidal neovascularization results in scarring in the macula and severe loss of vision. The combination of antipermeability and antiangiogenic activities manifested by Angl suggest that it has potential to be used as a therapeutic transgene for gene delivery approaches for these two disease processes.

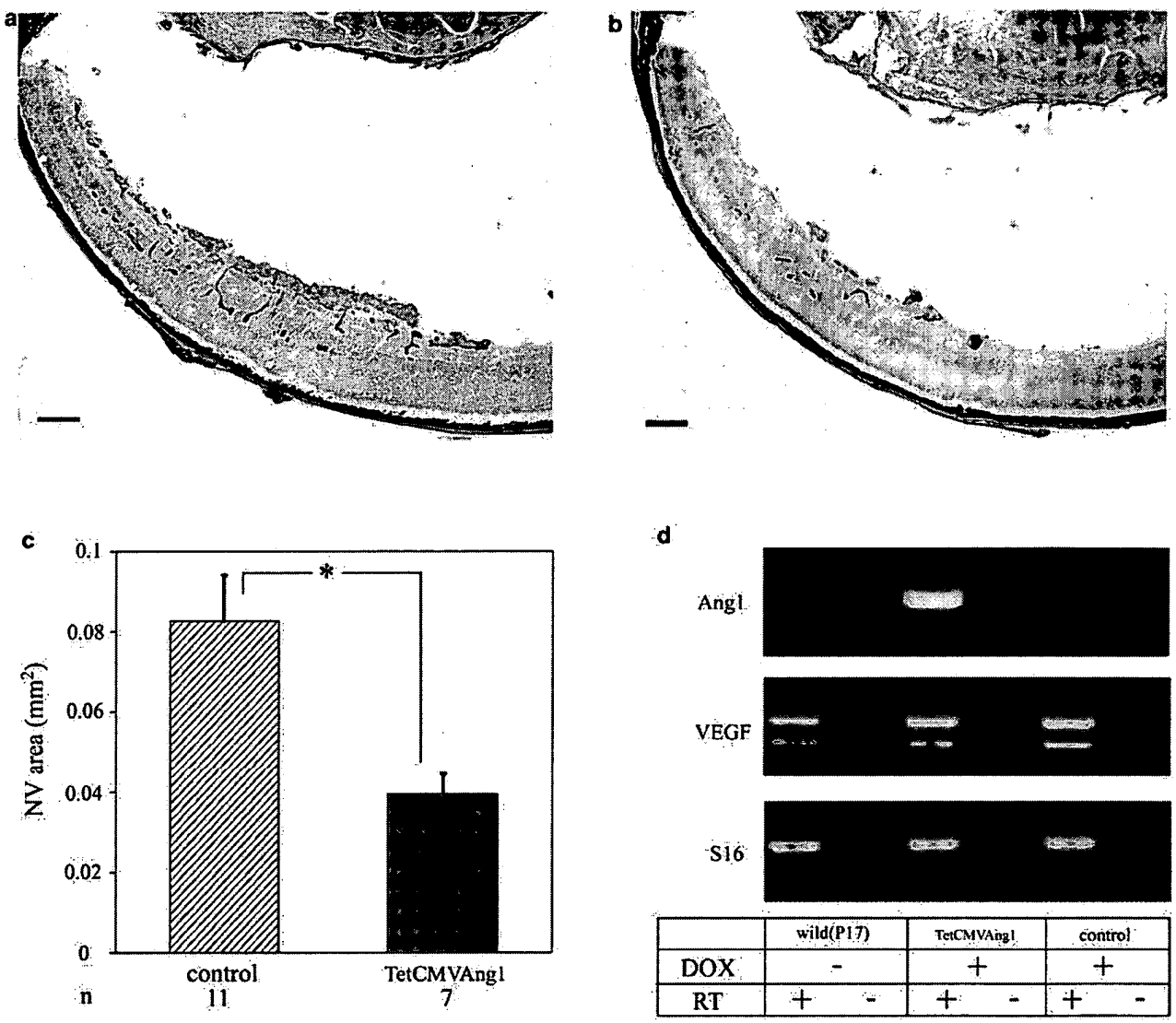

*Figure 4 Increased expression of Angl suppresses ischemia-induced retinal neovascularization (NV). Double hemizygous TetCMVAngl mice or littermate controls that did not carry both transgenes were placed in 75% oxygen at P7, and then removed to room air at P12 and given daily subcutaneous injections of 0.5 mg/g body weight of doxycycline. At P17, retinal sections stained with Griffonia simplicifolia lectin showed substantially more retinal neovascularization in control mice (a) compared to TetCMVAngl mice (b). Measurement of the area of neovascularization on the surface of the retina showed significantly more neovascularization in control mice, compared to TetCMVAngl mice (c). In doxycycline-treated TetCMVAngl mice with ischemic retinopathy, there was an increase in angl mRNA in the retina compared to controls with ischemic retinopathy, but there was no difference in vegf mRNA (d). *P = 0.0043 by Mann–Whitney U test. Bar = 100 μm.*

A recent study demonstrated that intravitreous injection of Angl* or systemic injection of an adenoviral vector expressing Angl decreased mild breakdown of the blood–retinal barrier that occurs acutely after the onset of hyperglycemia in rodents.[26] It is not clear that breakdown of the blood–retinal barrier occurring days after the onset of hyperglycemia has anything to do with diabetic retinopathy, which requires years of hyperglycemia to develop, but its suppression by Angl shows another situation in which Angl exhibits antipermeability effects in the eye. The above-mentioned study[26] did not examine the effect of Angl on ocular neovascularization, and to our knowledge the present study is the first demonstration that Angl suppresses ocular neovascularization.

Gene delivery offers potential treatment benefits because overexpression of an endogenous protein may have a greater likelihood of safety and tolerability compared to use of a drug that is not normally produced in the body. Also, gene delivery in the eye provides a means of achieving sustained local delivery. When considering a transgene as a therapeutic candidate, it is necessary to evaluate the effects of its prolonged expression in the retina to get an accurate assessment of its potential and rule out unexpected long-term toxicity. Transgenic mice with inducible expression of the transgene of interest provide a useful approach to evaluate the effects of that transgene independent of any possible vector-related effects. This study demonstrates that 8 weeks of expression of Angl in the retina at levels

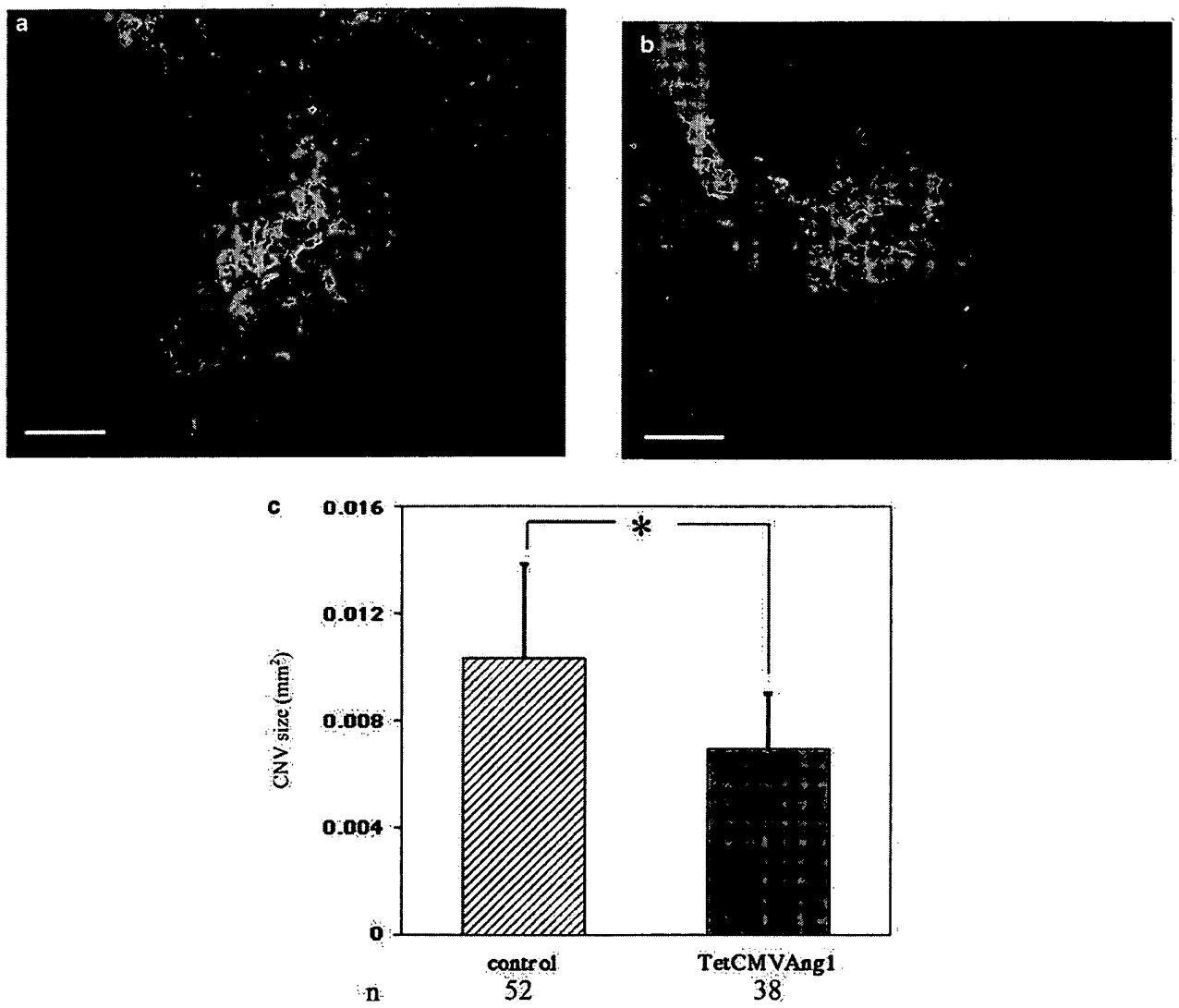

*Figure 5 Angl suppresses choroidal neovascularization (NV) at Bruch's membrane rupture sites. Adult TetCMVAngl mice and littermate controls that carried only one transgene had rupture of Bruch's membrane in three locations in each eye and then received 2 mg/kg of doxycycline in their drinking water. After 2 weeks, the mice were perfused with fluorescein-labeled dextran and the area of choroidal neovascularization at Bruch's membrane rupture sites was measured by image analysis. Compared to littermate control mice (a), TetCMVAngl mice (b) had significantly smaller areas of choroidal neovascularization (c). *P = 0.019 by Mann–Whitney U test. Bar = 100 μm.*

that suppress neovascularization and vascular leakiness has no identifiable deleterious effects on retinal vessels or retinal function. Future studies will investigate if safety and efficacy are maintained after 6–12 months of Angl expression in the retina, and will help to assess the feasibility of using AAV or lentiviral vectors for long-term expression of Angl as a possible treatment for ocular neovascularization and macular edema.

Materials and methods

Generation of double transgenic mice with inducible expression of Angl

A 2 kb *Xho*I fragment containing a full-length murine *angl* cDNA was subcloned into pKS+ (Stratagene) resulting in plasmid pKS+/mTL1. A 2.2 kb *Bam*HI/*Kpn*I fragment was excised from pKS+/mTL1 and ligated into pTRE1 (Clontech; GenBank Accession #: U89931) containing the *tetracycline response element* (*TRE*). After transformation, a clone with correct orientation of the *angl* fragment was identified by sequencing. Purified DNA was digested with *Aat*II and *Pvu*II yielding a 3.3 kb *TRE/angl/SV40 poly A* fusion gene. The fusion gene was purified and transgenic mice were generated as previously described.[27]

Mice were screened by polymerase chain reaction (PCR) of tail DNA using an upstream primer in the *TRE* domain (GAC CTC CAT AGA AGA CAC CG) and a downstream primer in the *angl* domain (CCT ATG TGA GTC AGA ATG GC) that amplify a 500 bp transgene-specific product. Tail DNA was obtained by overnight digestion of a 2 mm tail segment in 0.5 mg/ml proteinase K, 10 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 20 mmol/l ethylenediaminetetraacetic acid, and 2% Triton X-100 at 55°C. One *TRE/angl* transgenic founder

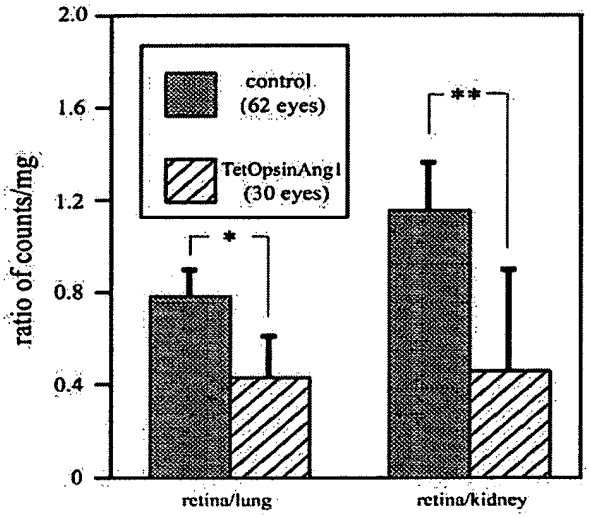

*Figure 6 Angl suppresses VEGF-induced leakage of [^{3}H]mannitol from retinal vessels. Adult TetOpsinAngl mice and littermate control mice that carried only one transgene were given 2 mg/ml of doxycycline in their drinking water for 2 weeks, and then all mice were given an intravitreous injection of 1 µl of 10^{-6} M VEGF. After 6 h, the retina to lung and retina to kidney leakage ratios were significantly less in TetOpsinAngl mice compared to littermate controls. *P = 0.011: **P = 0.019 by linear mixed model adjusted for multiple comparisons using Dunnett's method.*

was obtained and crossed with transgenic mice that express the reverse tetracycline transactivator specifically in photoreceptors (*rho/rtTA*, line D)[28] and a *rho/rtTA-TRE/angl* (TetOpsinAngl) double transgenic line was established. These mice were genotyped using the primers above to identify the *TRE/angl* transgene, and to identify the *rho/rtTA* transgene, the following primers were used, as previously described: forward, GTT TAC CGA TGC CCT TGG AAT TGA CGA GT; reverse, GAT GTG GCG AGA TGC CCT TGG AAT TGA CGA GT.[19,28]

To generate mice with doxycyline-inducible expression of Angl throughout the body, *TRE/angl* transgenic mice were crossed with transgenic mice in which the reverse tetracycline transactivator is under control of the CMV promoter (*CMV/rtTA* mice, Jackson Labs, Bar Harbor, ME, USA) to generate TetCMVAngl double transgenic mice. These mice were genotyped using the primers above to identify the *TRE/angl* transgene, and to identify the *CMV/rtTA* transgene, the following primers were used: forward, CGC TGT GGG GCA TTT TAC TTT AG; reverse, CAT GTC CAG ATC GAA ATC GTC.

RT-PCR

Adult TetOpsinAngl or TetCMVAngl mice were given drinking water containing 2 mg/ml of doxycycline and 5% glucose. After 2 weeks, mice were euthanized, eyes were removed, and retinas were dissected. Retinal RNA was isolated using Trizol (InVitrogen, Gaithersburg, MD, USA). Reverse transcription was carried out with ~0.5 µg of total RNA, reverse transcriptase (SuperScript II, Life Technologies, Gaithersburg, MD, USA), and 5.0 µM oligo dT primer. Aliquots of the cDNAs were used for PCR amplification using primers that specifically amplify a 402 bp fragment of murine *angl* (forward, AAG TGG CGA TTC TGT TGT TGA CC; reverse, CAC GAA GGA TGC TGA TAA CG). In some experiments, primers that specifically amplify a 557 bp fragment of murine *vegf* were used (forward, GCT CTA GAC CAT GAA CTT TCT GCT GCT TT; reverse, TAG GGA TCC TCA CCG CCT CGG CTT GTC ACA). Titrations were performed to ensure that PCR reactions were carried out in the linear range of amplification. Mouse S16 ribosomal protein primers (forward, CAC TGC AAA CGG GGA AAT GG; reverse, TGA GAT GGA CTG TCG GAT GG) were used to provide an internal control for the amount of template in the PCR reactions.

Immunohistochemistry

Mice were euthanized and eyes were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.6 containing 5% sucrose. After 30 min, corneas and lenses were removed and then the fixation was continued for 1 h. After washing with 0.1 M phosphate buffer/20% sucrose overnight, the eyecups were frozen in optimum cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN, USA). Ocular frozen sections (10 µm) were dried with cold air for 5 min, fixed in freshly prepared 4% paraformaldehyde in 0.05 M phosphate-buffered saline (PBS) at 4°C for 15 min, and rinsed with Tris-buffered saline (TBS) for 10 min. Endogenous peroxidases were inhibited by a 10 min incubation with 0.75% H_2O_2 in methanol. Antigen retrieval was used as described by Mitchell *et al*.[29] Briefly, sections were washed three times in distilled water and immersed for a few minutes in 0.01 M citrate buffer, pH 6.0 at room temperature. They were immersed for 2 min in 0.01 M citrate buffer preheated to 40°C, immersed for 2 min in buffer preheated to 60°C, heated in the microwave until the buffer boiled for 1 min, and then allowed to cool to room temperature for 1 h. After 10 min incubation in 50 mM TBS, nonspecific binding sites were blocked by incubating in 50 mM TBS/1% skim milk for 1 h at room temperature. Sections were incubated with 1:50 goat polyclonal anti-Angl (N18, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS/1% skim milk at 4°C overnight. Control sections were processed without primary antibody. After two rinses with TBS, sections were incubated for 1 h at room temperature with secondary antibody, 1:200 bovine anti-goat IgG-B (Vector Laboratories, Burlingame, CA, USA). After washing twice with TBS, sections were incubated for 30 min at room temperature with streptavidin phosphate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and then with HistoMark Red (Kirkegaard & Perry Laboratories). Sections were rinsed in TBS and deionized water, and dehydrated by passing them through a step gradient consisting of 70, 95, and 100% ethanol, xylene, and then mounted with Cytoseal XYL mounting medium (Richard-Alan Scientific, Kalamazoo, MI, USA). The stained sections were examined under a Nikon microscope and captured as digital files with a Nikon Digital Still Camera DXM1200 (Nikon Instruments Inc., NY, USA).

Histochemistry to stain vascular cells

To stain retinal vessels, frozen sections were stained with biotinylated *Griffonia simplicifolia* lectin B4 (GSA, Vector Laboratories) as previously described.[30] Briefly, paraformaldehyde fixed slides were incubated in methanol/

H_2O_2 for 10 min at 4°C, washed with 0.05 M TBS, pH 7.6 (TBS, pH 7.4), and incubated for 30 min in 10% normal porcine serum. Slides were incubated 2 h at room temperature with biotinylated GSA and after rinsing with 0.05 M TBS, they were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 min at room temperature. After a 10-min wash with 0.05 M TBS, slides were incubated with stable diaminobenzidine (ResGen, Huntsville, AL, USA) to give a brown section, counterstained with Contrast Blue (Kirkegaard and Perry), and mounted with Cytoseal (Stephens Scientific, Riverdale, NJ, USA).

Recording and analysis of ERGs

A previously described technique was used to record ERGs in Ang1-expressing and nonexpressing mice.[21] ERGs were recorded on four adult TetOpsinAng1 mice treated with 2 mg/ml of doxycycline in drinking water for 8 weeks and four untreated, age-matched TetOpsinAng1 mice. Mice were dark-adapted for a standardized 12 h period overnight and ERG recordings were performed using the Espion ERG Diagnosys (Diagnosys LLL, Littleton, MA, USA). All manipulations were carried out with dim red light illumination. Beginning the same time each morning, mice were anesthetized by intraperitoneal (IP) injection of 25 µl/g body weight of Avertin (Aldrich, Milwaukee, WI, USA) diluted 1:39 in PBS. Each cornea was anesthetized with a drop of 0.5% proparacaine hydrochloride (Alcon Labs, Inc., Forth Worth, TX, USA) and pupils were dilated with 1% mydriacyl ophthalmic solution (Alcon Labs). Mice were placed on a pad heated to 37°C and platinum electrodes were placed on each cornea after application of gonioscopic prism solution (Alcon Labs). The reference electrode was placed subcutaneously in the anterior scalp between the eyes, and the ground electrode was inserted into the tail. Electrode impedance was balanced for each eye pair measured. The head of the mouse was placed in a standardized position in a ganzfeld bowl illuminator that assured equal illumination of the eyes. Simultaneous recordings from both eyes were made for 11 intensity levels of white light ranging from −3.00 to +1.40 log cd-s/m^2. The Espion ERG machine measures the ERG response six times at each flash-intensity and records the average value.

Mice with oxygen-induced ischemic retinopathy

Ischemic retinopathy was produced by a previously described method.[22] Double hemizygous TetCMVAng1 mice were crossed and on the day of birth, mothers were started on 2 mg/ml of doxycyline in their drinking water. Litters were placed in 75±3% oxygen at P7. Incubator temperature was maintained at 23±2°C, and oxygen was measured continuously with an oxygen analyzer. Between P12 and P17, the mice were kept in room air and were given daily subcutaneous injections of 0.5 mg/g body weight of doxycycline. At P17, the mice were genotyped by PCR of tail DNA, euthanized, and eyes were frozen in OCT embedding compound. Serial frozen sections were stained with GSA, counterstained with eosin, and the area of retinal neovascularization was measured for each eye by image analysis with the investigator masked with respect to genotype.

Mice with choroidal neovascularization due to laser-induced rupture of Bruch's membrane

Choroidal neovascularization was generated by modification of a previously described technique.[23] Adult double hemizygous TetOpsinAng1 mice and controls that carried only one of the transgenes had rupture of Bruch's membrane in three locations in each eye with laser photocoagulation. Mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and the pupils were dilated with 1% tropicamide. Three burns of 532 nm diode laser photocoagulation (75 µm spot size, 0.1 s duration, 120 mW) were delivered to each retina using the slit lamp delivery system of an OcuLight GL Photocoagulator (Iridex, Mountain View, CA, USA) and a hand held cover slide as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. Production of a bubble at the time of laser, which indicates rupture of Bruch's membrane, is an important factor in obtaining CNV,[23] so only burns in which a bubble was produced were included in the study. On the day of laser treatment, the control mice and the double transgenics started to receive 2 mg/ml of doxycycline in their drinking water. After 2 weeks, the mice were anesthetized and perfused with 1 ml of PBS containing 50 mg/ml of fluorescein-labeled dextran (2×10^6 average mw, Sigma, St Louis, MO, USA) as previously described,[31] and the area of choroidal neovascularization at Bruch's membrane rupture sites was measured on choroidal flat mounts by image analysis.[32] Eyes were removed and fixed for 1 h in 10% phosphate-buffered formalin. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup. Radial cuts (4–7, average 5) were made from the edge to the equator and the eyecup was flat mounted in Aquamount with the sclera facing down. Flat mounts were examined by fluorescence microscopy on an Axioskop microscope (Zeiss, Thornwood, NY, USA) and images were digitized using a three-color CCD video camera (IK-TU40A, Toshiba, Tokyo, Japan) and a frame grabber. Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA) was used to measure the total area of hyperfluorescence associated with each burn, corresponding to the total fibrovascular scar. The areas within each eye were averaged to give one experimental value per eye for plotting the areas in the figures.

Measurement of VEGF-induced leakage of [^{3}H]mannitol in the retina

Adult (4–5 week old) double hemizygous TetOpsinAng1 mice and controls that carried only one of the transgenes were given 2 mg/ml of doxycycline in their drinking water. After 2 weeks, mice were given an intravitreous injection of 1 µl of 10^{-6} M human VEGF (R & D Systems, Minneapolis, MN, USA). After 6 h, mice were given an intraperitoneal injection of 1 µCi/g body weight of [^{3}H]mannitol (New England Nuclear, Boston, MA, USA). After 1 h, the retina, the left lobe of the lung, and the left kidney were removed and placed into preweighed scintillation vials. A 1 ml measure of NCS II solubilizing solution (Amersham, Chicago, IL, USA) was added to each vial and they were incubated overnight at 50°C. The solubilized tissue was decolorized with 20% benzoyl peroxide, and 5 ml of Cytoscint ES (ICN,

Aurora, OH, USA) and 30 µl of acetic acid were added. Radioactivity was counted and the retina to lung and retina to kidney leakage ratios were measured as previously described.[25]

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